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MEMORANDUM

TO: Commandant
Academy of Health Sciences, U.S. Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234-6100

FROM: K. O. Smith, Ph.D.

SUBJECT: Annual Report

Please find enclosed the annual report for Contract No. DAMD-17-86-C-6285, by Kendall O. Smith, PI.

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THE ROLE OF CYTOMEGALOVIRUS AS A COFACTOR IN THE DEVELOPMENT OF ARC-AIDS

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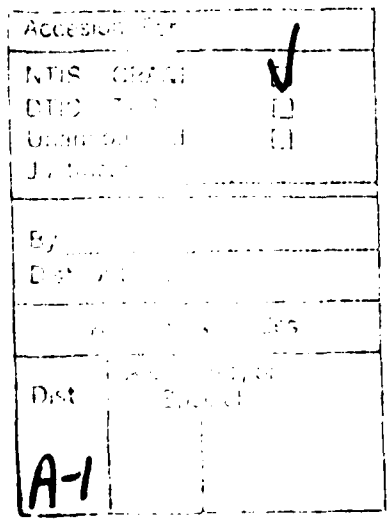

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A. Statement of the problem

The first phases of this project involved collection of sera, saliva and urine for storage and retrieval for antibody and virus isolation studies. Initial studies allowed development and modification of methodology and the identification of subjects in Groups 1, 2 and 3. The second phase of the work is now well under way, i.e., to obtain data aimed at determining whether CMV may play a role as a triggering cofactor with HIV to initiate rapid progression of disease. Time is required for significant numbers of the 600 subjects to progress; it will be several years before a sufficiently large number will have progressed to be adequate for valid statistical analysis. ~~The following~~ report describes some aspects of the study which we have been able to address recently, while we wait for disease progression to occur in sufficient numbers of subjects.

Most of the information presented in previous quarterly reports is not repeated in this report. Data has been generated on patterns of herpes simplex virus antibody production, as well as the CMV studies. This is because herpes simplex virus (HSV), particularly type 2, may be involved significantly in the initial venereal infection by HIV (through and from herpetic ulcers) and both CMV and HSV are now known to "trigger" HIV out of a latent state *in vitro*. The HSV work we now report was relatively easy to do and was done at no additional cost to the project; the methodology for CMV and HSV work were the same.

The total number of different HIV-seropositive subjects we have studied is slightly over 600, which is about 68% of the seropositive subjects coming to Wilford Hall Hospital for evaluation. Thirty-four (5.7%) of the approximately 600 subjects were seronegative by Western blot (WB) on entry into the study. Six of these 34 originally CMV seronegative subjects have seroconverted, showing banding by WB.

One of the major aspects of our original proposal was to compare three groups of subjects in regard to rate of disease progression (by the WR classification):

Group 1 - CMV seropositive subjects;

Group 2 - CMV seronegative subjects who remain seronegative; and

Group 3 - CMV seronegative subjects who seroconvert during the course of our study (indicating primary infection)

B. Results

(1) Question: What changes occured in CMV and HSV-1-specific IgG and neutralizing antibody concentrations among HIV-1-infected subjects who advanced two or more Walter Reed classification numbers during the course of this study?

Earlier and later (paired) sera had been frozen and were available on each of 18 subjects who were observed to advance 2 or more WR numbers as of 12/88; these sera were titrated by serial dilution to end-point by WB and by neutralization (plaque reduction assay) against both CMV and HSV-1. The purpose was to study by WB the antibodies directed against each of the more than 25 viral proteins of each virus, and, by virus neutralization, obtain data concerning the presence of biologically significant antibodies, e.g. those which neutralize these two viruses. Tables I and II show some of the results from these studies in 18 subjects who changed an average of 2.9 WR classification units between the two serum collections.

(a) Cytomegalovirus antibodies. 14/18 subjects had no significant (meaning 3.2-fold or less) antibody titer changes measurable by WB end-point determination (highest serum dilution giving visible bands). 3/18 had 10 to 32-fold WB titer increases while 1/18 had a 10-fold decrease in WB titer. The one subject with a decreased WB titer (#347) also had a 32-fold decrease in CMV-specific neutralizing antibody titer. The neutralization titers in most cases paralleled WB titers at a lower level, by an average of about 1.5 log units (32-fold), i.e., the WB assay was about 32-fold more sensitive than the viral neutralization (plaque reduction) assay for CMV antibodies. The mean WB titers of 18 paired sera were: for early sera, $10^{3.30}$; for later sera, $10^{3.50}$ (no significant difference). Likewise, there was no significant change in the means of CMV neutralizing antibody titers. It is concluded that there were no overall titer changes in CMV-specific IgG in HIV-1-infected subjects who progressed in WR classification an average of 2.9 units.

An interesting change in WB patterns seemed to be associated with either gain (Figure 1, left) or loss (Figure 1, right) of CMV antibodies in subjects #218 and #347, respectively. As with HSV-1 antibody patterns seen in Figure 2, loss or gain of significant quantities of CMV antibodies was visually reflected in substantial band pattern changes in western blots.

(b) Herpes simplex virus type 1 antibodies. By WB, 13/18 subjects had no significant (3.2-fold or greater) antibody titer change to HSV-1; 3/18 had a 10- to 32-fold increase, and 2/18 showed a substantial decrease (32- to 100-fold) in WB antibody titer. Subject #347 is the

one who had a substantial decrease in CMV antibody titer (Table I), measurable both by WB and by neutralization assays. This subject also displayed more than a $10^{2.5}$ (320)-fold drop in neutralizing antibody against HSV-1 (Table II).

In contrast with the WB data, however, virus neutralization data showed more significant increases in HSV-1 antibody from earlier to later WR stages. Similar to the WB data for CMV antibodies, the WB assays for HSV-1 antibodies in earlier serum collections were an average of $10^{1.65}$ (45)-fold more sensitive than neutralization assays. Later serum collections (from more advanced subjects), however, showed an average of only $10^{0.84}$ (6.9)-fold greater sensitivity of the WB assay over the virus neutralization assay. This substantial increase in the biological activity (neutralizing ability) of HSV-1 antibody in sera from advancing subjects is seen most clearly in patients #76 and #102 (Table II). In these cases, the HSV-1 neutralizing antibody titers in later serum collections actually exceeded their WB titers. Therefore, virus neutralization assays demonstrate the ability of some subjects with advancing disease to produce large quantities of biologically functional antibodies against HSV-1.

The data shown in Tables I and II reflect only end-point dilutions giving visible bands of any kind by WB analysis, and do not reveal possibly interesting changes in patterns in banding when earlier and later serum antibodies are compared (e.g., see Figure 2, HSV-1 antibody pattern changes; Figure 1 shows typical examples of CMV antibody patterns in earlier and later sera analyzed by WB),

Subjects #218, #157 and #225 each had significant increases in virus neutralizing antibodies from the times of earlier to later serum collections (Table II). Marked increases can be seen in the strength and number of WB bands, particularly in the lower molecular weight viral protein regions. In contrast, subjects #347, #321 and #571 each showed substantial losses of HSV-1 neutralizaing antibodies between the earlier and later serum collections. In these 3 subjects, there were also marked decreases in both the strength and the numbers of WB bands, again, particularly in the lower molecular weight viral protein regions. It will be interesting to follow the clinical courses of patients showing substantial waning and increasing in CMV and HSV-1 antibodies to determine whether clinical course is related to the biological "quality" and quantities of antiviral globulins the subjects produce.

TABLE I. CHANGES IN CYTOMEGALOVIRUS-SPECIFIC IgG AND NEUTRALIZING ANTIBODY CONCENTRATIONS IN HIV-1-INFECTED SUBJECTS WHO ADVANCED TWO OR MORE WALTER REED (WR) CLASSIFICATION NUMBERS DURING THIS STUDY

Patient No.	WR Classification Number	Log 10-IgG Titer (Western Blot)*	Log 10-Fold IgG Titer Change	Log 10-Neut. Ab Titer (Plaque Reduction)*	Log 10-Fold Neut. Ab Titer Changes
76	2 → 5	3.5 → 3.5	None	2.0 → 2.5	↑0.5
218	1 → 3	2.5 → 4.0	↑1.5	1.0 → 2.0	↑1.0
201	1 → 4	3.0 → 4.0	↑1.0	<1.0 → 2.0	↑ >1.0
178	1 → 5	3.0 → 3.0	None	1.0 → <1.0	<
347	1 → 3	4.0 → 3.0	↓1.0	2.5 → <1.0	↓ >1.5
102	1A → 5A	2.5 → 3.0	↑0.5	2.0 → 2.5	↑0.5
124	1A → 3A	2.0 → 2.5	↑0.5	<1.0 → <1.0	None
244	1A → 3A	3.0 → 3.0	None	2.0 → 3.0	↑1.0
80	1 → 5	3.0 → 3.0	None	1.5 → 1.5	None
157	1 → 4	3.0 → 2.5	↓0.5	<1.0 → to be done	to be done
170	2 → 4	3.5 → 4.0	↑0.5	2.0 → 2.0	None
391	2 → 5	4.5 → 4.5	None	2.5 → 2.5	None
321	1A → 4A	4.0 → 4.0	None	to be done → 1.5	to be done
225	4A → 6B	4.0 → 4.0	None	<1.0 → to be done	to be done
604	1A → 3A	4.0 → 4.0	None	1.5 → 1.3	↓0.2
195	1A → 5A	2.5 → 3.5	↑1.0	<1.0 → <1.0	↑
222	1A → 6B	3.5 → 3.5	None	1.0 → <1.0	↓
571	4A → 6B	4.0 → 4.0	None	2.5 → <1.5	↓1.0
$\bar{x}$	1.5 → 4.4	3.30 → 3.50	↑ 0.20	1.79 → 1.94	↑ 0.15

*Serial dilution end-points.

TABLE II. CHANGES IN HERPES SIMPLEX VIRUS-SPECIFIC IgG AND NEUTRALIZING ANTIBODY CONCENTRATIONS IN HIV-1-INFECTED SUBJECTS WHO ADVANCED TWO OR MORE WALTER REED (WR) CLASSIFICATION NUMBERS DURING THIS STUDY

Patient No.	WR Classification Number	Log 10-IgG Titer (Western Blot)*	Log 10-Fold IgG Titer Change	Log 10-Neut. Ab Titer (Plaque Reduction)*	Log 10-Fold Neut. Ab Titer Changes
76	2 → 5	3.0 → 3.0	None	1.9→4.0	↑2.1
218	1 → 3	3.5 → 4.0	↑0.5	2.7→3.5	↑0.8
201	1 → 4	3.5 → 4.0	↑0.5	2.7→3.5	↑0.8
178	1 → 5	3.0 → 3.0	None	2.4→2.2	↓0.2
347	1 → 3ϐ	4.5 → 2.5	↓2.0	>3.5→1.0	↓>2.5
102	1A → 5A	3.0 → 3.5	↑0.5	<1.0→>4.0	↑3.0
124	1 → 3	3.0→ 2.5	↓0.5	<1.0→1.3	↑0.3
244	1A → 3A	4.0 → 4.0	None	2.1→2.6	↑0.5
80	1 → 5	3.5 → 3.5	None	2.0→2.0	None
157	1 → 4	3.0 → 4.5	↑1.5	1.8→3.3	↑1.5
170	2 → 4	3.5 → 4.0	↑1.5	2.1→2.0	↓0.1
391	2 → 5	2.0 → 2.0	None	2.5→2.5	None
321	1A → 4A	4.0 → 4.0	None	3.7→2.5	↓1.2
225	4A → 6B	3.0 → 4.0	↑1.0	1.6→3.4	↑1.8
604	1A → 3A	3.5 → 3.5	None	2.7→2.6	↓0.1
195	1A → 5A	3.0 → 3.5	↑0.5	1.9→2.1	↑0.2
222	1A → 6B	3.5 → 3.5	None	<1.0→<1.0	None
571	4A → 6B	4.5 → 3.0	↓1.5	4.0→2.3	↓1.7
	1.5 → 4.4	3.39 → 3.44	↑0.05	1.74→2.60	↑0.84

*Serial dilution end-points.

(2) Question: What is the prevalence of HSV-1 and HSV-2 antibodies in HIV- seropositive and -seronegative individuals?

A commercially available kit (Herpelisa, Whittaker Bioproducts) for differentiating HSV-1 and HSV-2 antibodies proved unsatisfactory (too variable, insensitive) in our hands, so we used the more sophisticated WB analysis to study age and sex matched HIV-seropositive and -seronegative subjects for herpesvirus antibodies. All tests were coded, then the WB strips on matched sera and patient sera were run in alternating wells, dried, photographed and read blindly. In most cases, group- and type-specific HSV antigen bands (Figure 3) were clearly differentiated. Six of 92 sera studied gave equivocal results concerning differentation of HSV type, so were deleted from the study. The results, shown in Table III and Figure 3, indicate that the prevalence of HSV-2 antibodies is much greater in the HIV seropositive group (77%) than in the matched control group (30%); $p = < .01$. Moreover, none of the HIV-seropositive individuals were HSV-1 and HSV-2 seronegative. Prevalence of HSV-1 antibodies in the two groups were about the same. This study suggests that changes in HSV-2 antibodies during the course of AIDS disease progression may be more important to follow than HSV-1 antibodies. Therefore, certain of the subjects described in Table II and Figure II (showing HSV-1 antibody changes) are being studied further by WB and neutralizing antibody titrations to follow changes in HSV-2 antibodies as they progressed in WR classification during the period from the first bleeding to the last. It appears that some subjects who have advanced rapidly in WR classification are progressively losing their CMV and HSV antibodies, while others maintain or increase antibodies to both viruses. This may herald capability or incapability to maintain these viruses in a steady (equilibrium) state with host defenses and the roles of these viruses as opportunists.

TABLE III. Herpes simplex virus (HSV) type 1 and 2 antibodies in the sera of HIV-seropositive and seronegative individuals.

HIV-Serological Status	HSV-1 Serological Status		HSV-2 Serological Status		HSV-1 and HSV-2 Serological Status
	+	−	+	−	−
+ (n = 43)	27 (63%)	16 (37%)	33 (77%)	10 (23%)	0 (0%)
− (n = 43)	28 (65%)	15 (35%)	13 (30%)	30 (70%)	6 (14%)

(3) Question: Are CMV isolates significantly different antigenically from a prototype CMV strain and from each other?

Many different CMV isolates have now been co-electrophoresed, each with molecular weight standards and Towne (prototype) strain CMV, then studied in detail by WB to detect strain differences in antigenic composition when reacted with pooled high-titered CMV antibodies. These studies, in summary, indicate that different CMV strains are qualitatively almost identical in their antigenic mosaics, yet can vary in the relative quantities of certain major antigens produced. For example, there is wide variation in the production of the 140Kd band CMV protein, yet all strains we have studied thus far possessed at least a small amount of this protein. Determining whether variations in the relative quantities of major viral proteins are characteristics (under the genetic control) of particular strains or are functions of viral growth conditions have been studied. Plainly, it would be detrimental to CMV serologic investigations if major CMV proteins were in low concentrations in antigens used for antibody testing.

Minor antigenic variations between strains were studied using the patients' own antibody and CMV strain in WB analysis. The rationale for this approach is that if a CMV strain should have a unique antigenic protein, the only way to demonstrate it would be by use of the unique antibodies from an individual infected with that particular strain. The results of our studies suggest that all CMV strains we have isolated (over 50) are qualitatively almost identical, variations occurring only in the quantity of each viral protein produced on passage of that pariticular isolate. Published reports addressing this issue have presented data using pools of sera, not the serum from the subject from whom the isolate came. Therefore, we reason that some of the reports on antigenic variability between strains are probably

in error, attributable in part to quantitative differences in the proteins produced by different CMV strains under variable passage conditions.

One complication in interpretation of this kind of data is that most CMV isolates we have obtained came from individuals with low CMV-specific serum IgG titers, therefore antibodies to any unique antigenic proteins would be hard or impossible to detect by WB because of their small quantity. Moreover, the number of bands and the quantity of each protein band in WB can vary significantly in different passages of prototype CMV strains, even when conditions of virus passage are maintained as nearly constant as possible. With possibly one or two minor exceptions, we have no evidence for unique antigenic proteins in any of our fresh isolates; they all seem qualitatively identical to the prototype Towne strain.

(4) Question: Are WR4, 5 and 6 subjects more likely to shed CMV in their salivas than WR-1 subjects?

Thirty saliva specimens from WR-4, 5 and 6 subjects and 30 from WR-1 subjects were withdrawn from storage; all had been frozen with 10% DMSO and 5% calf serum, then kept at -70°C for several months before thawing and culturing for CMV. After centrifugal inoculation of specimens on MRC-5 cells and observation of cultures for 23 days, 8/30 (26%) and 2/30 (7%) were found to contain infectious CMV (from WR-4, 5 and 6 vs. WR-1 subjects, respectively). It is concluded, therefore, that increased rate of CMV shedding in saliva is associated AIDS progression. These studies on frozen-thawed-cultured specimens confirm earlier conclusions based on specimens never frozen-thawed (cultured immediately on receipt). Increased CMV shedding and higher CMV-specific IgM production are both associated with advancing disease.

(5) Question: Is CMV-specific antibody secreted into the saliva and, if so, does this affect the isolation of infectious virus from saliva specimens?

Saliva and plasma from the same subjects were serially diluted and tested by WB for antibodies to CMV. It was found that CMV-specific IgG was usually present in saliva of subjects having plasma antibodies at a concentration at or above 3,000; the mean ratio of concentrations of CMV-specific IgG in plasma and saliva was about 3,200. IgA class of CMV-specific antibodies was not detectable by WB.

The effect of CMV antibodies in saliva upon our ability to isolate infectious CMV has been, and is now under study, with a view toward finding means for minimizing the neutralizing potential of such antibodies during the process of infectious virus isolation. We are relatively certain that CMV-specific IgG in the saliva precludes routine isolation of virus from this specimen if the IgG titer is above about 1:10-1:30 by WB. We have been successful in a strategy of blocking the neutralizing effect of CMV antibody by adding heat-inactivated CMV antigen to mock-infected fluid containing known amounts of CMV neutralizing antibody, almost 100% recovery of virus being achieved. To continue this strategy, we plan to place heat-inactivated CMV antigen in freshly collected saliva immediately after collection and study the efficiency of CMV isolation from such "blocked" and "unblocked" specimens. We predict a much higher CMV isolation rate if the CMV-specific neutralizing antibodies in saliva specimens are routinely blocked.

Figure 1. Changes in WB banding patterns of CMV antibodies in individuals with advancing disease (from WR-1A to WR-3A or B).

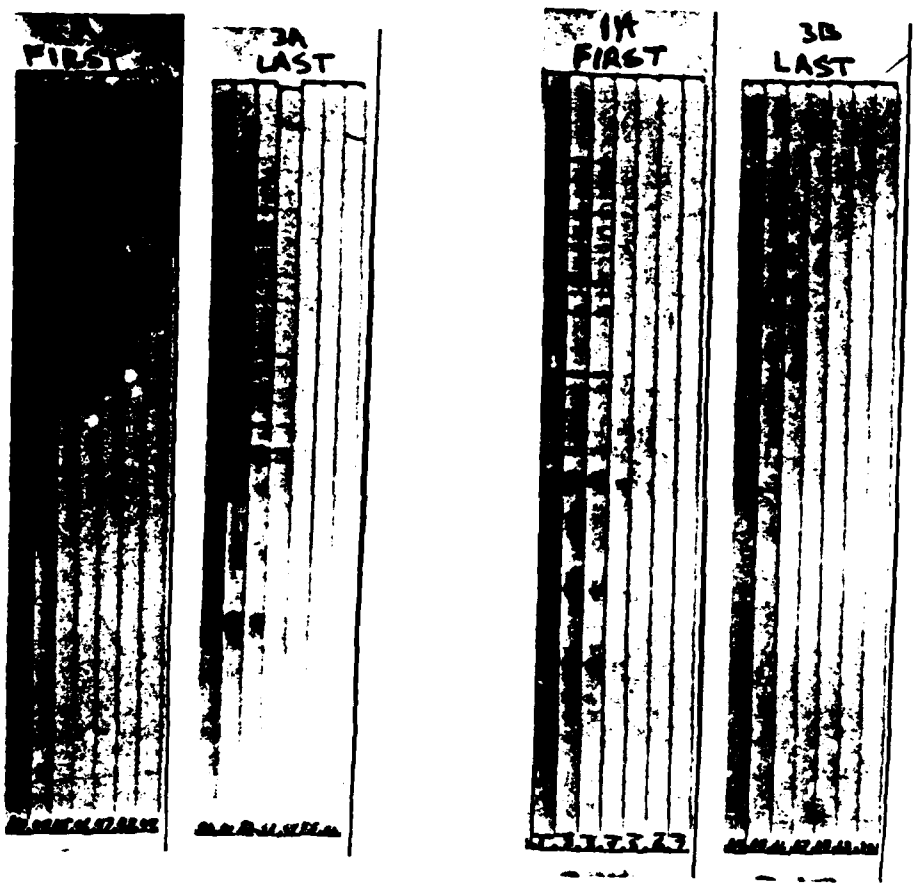

Figure 2. Changes in WB banding patterns of HSV antibodies in individuals with advancing disease (from WR-1A to WR-3A or B).

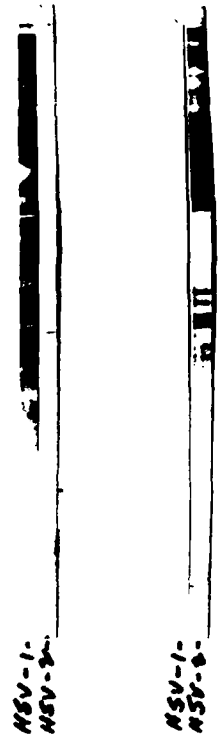

Figure 3. Differentiation of HSV-1 and HSV-2 specific antibodies by WB. On the left is an example of a subject with HSV-1 type specific antibodies, showing weak cross-reactions against group antigens in the HSV-2 strip. On the right is an example of a subject with HSV-2 type specific antibodies, showing weak cross-reactions against group antigens in the HSV-1 strip.